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Evaluation of Association of MicroRNA-122 with Histological Severity of Recurrent HCV Infection in Liver Transplant Recipients

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EVALUATION OF ASSOCIATION OF MICRORNA-122 WITH HISTOLOGICAL SEVERITY OF RECURRENT HCV INFECTION IN LIVER TRANSPLANT RECIPIENTS

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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LIST OF ABBREVIATIONS

AB	applied biosystems
ALT	alanine aminotransferase
ANOVA	an analysis of variance
B2M	beta-2-microglobulin
BCAP31	B cell receptor-associated protein 31
Ca2+	calcium
CCHCR1	coiled-coil alpha-helical rod protein 1
CD4	cluster of differentiation 4
cDNA	complementary DNA
C. elegans	Caenorhabditis elegans
CFTR	cystic fibrosis transmembrane conductance regulator
CHC	chronic hepatitis C
CT	critical threshold
ΔCT	delta critical threshold
°C	degrees celcius
DEG	differentially expressed genes
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum

ERAD	endoplasmic reticulum associated degradation
Exp5	Exportin 5
FFPE	formalin-fixed paraffin-embedded
GUSB	glucuronidase-beta
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCVrec	hepatitis C virus recurrence
Huh 7	human hepatoma cell line
IFN-γ	interferon gamma
IL-2	interleukin-2
IRES	internal ribosomal entry site
LT	liver transplantation
MDx	molecular diagnostics
MGB	minor groove binder
MHC	major histocompatibility complex
µl	microliter
µm	micrometer
min	minute
miRNA	microRNA
mRNA	messenger RNA
NFQ	nonfluorescent quencher
ng	nanogram

non-TX	non-transplanted
nt	nucleotide
NTC	no template control
OLT	orthotopic liver transplantation
ORF	open reading frame
PCR	polymerase chain reaction
pol	polymerase
post-TX	post-transplantation
post-LT	post-liver transplantation
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RT	reverse transcription
RT-PCR	real-time polymerase chain reaction
SVR	sustained viral response
TE/Tris-HCl	(hydroxymethyl) aminomethane hydrochloride
Th1	T-helper 1
TMDs	transmembrane domains
Treg	regulatory T-cells
UNG	uracil-N-glycosylase
UTR	untranslated region

Abstract

EVALUATION OF ASSOCIATION OF MICRORNA-122 WITH HISTOLOGICAL SEVERITY OF RECURRENT HCV INFECTION IN LIVER TRANSPLANT RECIPEINTS

By Jihee Lacey Suh

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

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Hepatitis C virus recurrence (which is defined by detection of HCV RNA in serum) in post-transplanted liver is universal but the progression of infection remains unpredictable, varying from case to case. It has been estimated that 75%-80% of the HCV recurrence patients will suffer chronic hepatitis C infection and up to a third of them will progress into the development of fibrosis and cirrhosis within 5 years post-transplantation. Therefore, finding ways to predict early on the progression of fibrosis can contribute to better prognoses. Recent literatures have mentioned that the hepatitis C virus relies on the

host microRNA-122 (miR-122) for assistance in replication of the viral genome in hepatocytes. Experimental depletion of miRNA-122 in the cell line Huh 7 has shown up to an 80% decrease in HCV whereas an increase of miRNA-122 has shown an increase of HCV. Since miRNAs are known to have numerous indirect roles by the binding of the target messenger RNAs (mRNAs) and repressing the expression of their proteins, we hypothesized that the elucidation of associations between miRNA-122 and the histological severity in HCV recurrence post-liver transplantation might serve as a biomarker in predicting the outcome of HCV recurrence severity in patients. We also evaluated the expression levels of BCAP31 (a predicted target of miRNA-122), and CD4 (T cell surface molecules involved in immune response) among the HCV recurrence severity groups.

RNA samples were isolated from FFPE liver samples from patients with HCV recurrence post-transplantation, and Reverse Transcription and TaqMan Real-Time PCR were carried out for qualitative analysis. We did not see any association between the levels of miRNA-122 expression and severity of HCV recurrence, but we did find a positive correlation between the miRNA-122 expression and the HCV viral load in Group 3 (Severe) at time of HCV recurrence, which supports previous studies of the role of miRNA-122 in HCV replication. We did not find any associations between the expression of BCAP31 and the severity of HCV recurrence but we did discovery an inverse relationship between miRNA-122 and BCAP31 in Group 3 (Severe) at time of HCV recurrence, confirming our assumption of miRNA:mRNA interaction. Also, we did find CD4 expression being statistically significant between Group 1 (Benign) versus Group 3

(Severe), which may support the hypothesis that strong, adequate CD4+ T-cell response is associated with better outcome post-liver transplantation.

CHAPTER 1 Introduction

Hepatitis C Virus Infection

Hepatitis C Virus (HCV) has originally been identified in 1989 as a positive-stranded RNA flavivirus which according to the World Health Organization infects an estimate of 170 million people worldwide (1-3). HCV is known to have a rapid mutation rate and is classified into 6 genotypes and over 100 subtypes varying among people (3, 4). Majority of HCV-infected Americans who were born between 1945 and 1964 acquired the virus through either needle sharing involved in recreational drug use or by blood transfusions that were received before 1992 due to the lack of HCV antibody screening. Other potential risk factors to HCV infection are long-term hemodialysis and frequent blood exposure in health care environments (5, 6).

HCV is highly persistent and 75% of HCV-infected patients develop chronic liver diseases (7, 8). Currently there are few therapeutic options such as, combination therapy using the cytokine peginterferon-α which is injected subcutaneously once a week and the nucleoside analogue ribavirin which is taken orally twice daily to achieve a sustained viral response (SVR) as conventional treatment for chronic hepatitis C (CHC) infection. Patients with HCV genotype 2 or 3 are known to have about 80% or higher success rates to treatment after 12-24 weeks, whereas genotype 1 and 4 have only 50% response rate to

treatment after 24-48 weeks (8). The length of antiviral therapy is not only expensive and the side effects unpleasant, but there is no guarantee that the outcome will be successful for everyone (8). No vaccines are currently available either for prevention (1, 7).

In the U.S., liver transplantation (LT) is often performed in response to end-stage liver disease caused by CHC infection (4, 9, 10). However, transplantation does not end the battle of HCV infection because re-infection within the new liver is almost universal (9-11). Cases demonstrate approximately 10-41% of the liver-transplant recipients will again develop cirrhosis in the new liver within 5-10 years (8). Both the host and the viral factors are found to play an interchanging role in CHC infection, possibly permitting genotypic influences but the molecular pathways regarding the actions of host factors within the HCV replication presently remains unclear (12, 13).

Hepatitis C Virus

The pathogen's genome is composed of 9,600 nucleotides (nt) in length with a single open reading frame (ORF) that encodes for a roughly 3,000 amino acids long precursor protein which eventually gets cleaved co- and post-translationally into three structural (core, E1, E2) and seven non-structural proteins (p7, NS2-NS5B) (14-16). Each of the 10 different products has its specific roles and functions that play a vital part in the HCV life cycle (1, 17). The viral 3'-untranslated region (UTR) and the ribonucleic acid (RNA) stem-loop structures I and II within the 5'-UTR are known to be involved in RNA replication whereas the stem-loops II-IV makes up the internal ribosome entry site (IRES).

The IRES allows ribosome to bind directly onto this region without translation initiation factors, assisting in control of translation (18).

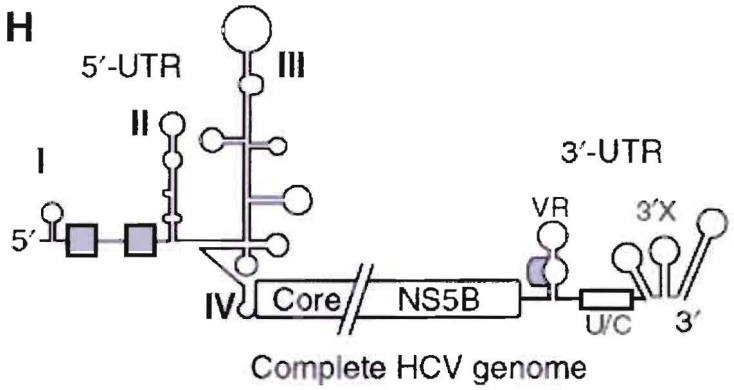


Figure-1: The full-length HCV RNA genome. (Henke, the EMBO Journal 2008) (18).

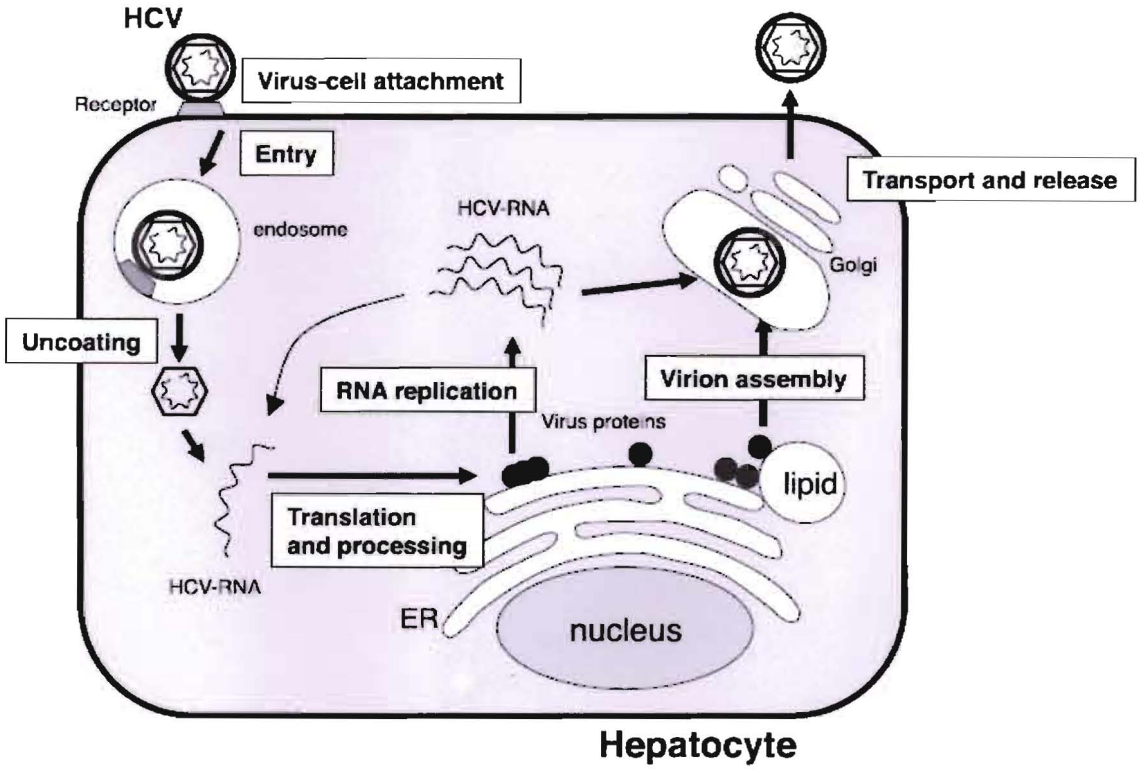


Figure-2: Life cycle of HCV. (Sakamoto, Journal of Gastroenterology 2009)(19).

Liver Cirrhosis and Chronic Hepatitis C Virus infection

HCV infection is known to be the major leading cause of liver cirrhosis and hepatocellular carcinoma (HCC) (8). About 10-20% of the patients infected with HCV will develop cirrhosis within the 10-30 years time period and within that group, about 1-5% of the patients will go on developing HCC (8). Prior studies have shown gene expression profiles of HCV cirrhosis displaying a pattern of over-expressed genes involved with the inflammatory response and a pattern of under-expressed genes involved in the cell cycle control in the late stages of cirrhosis (20). These results could partially explain why some patients progress forth to the development of cancer in the later stages of HCV cirrhosis. Also, expression of genes that are responsible for normal protein synthesis were noticeably different in cirrhotic livers (21). These results may correlate to the findings of many cirrhotic patients demonstrating impairment of protein synthesis in their livers which can lead to abnormal blood coagulation and uncontrolled colloid osmotic pressure (21).

Unfortunately, approximately 85% of the infected patients are not able to clear the virus (22, 23). Although the HCV RNA can be detected within 7-10 days after initial exposure and their antibodies can be detected within 49-70 days of infection, due to the mild and non-specific symptoms of this disease, primary infection is often gone unnoticed. Therefore, it is often diagnosed accidentally, which contributes to the high incident of chronic infections versus acute infections, and delayed treatment will most likely result in further complications (8).

HCV Recurrence Post-Liver Transplantation

Hepatitis C virus recurrence (HCVrec) after orthotopic liver transplantation (OLT) is nearly universal (10, 11, 24-27). Recurrent HCV infection is defined as detection of HCV RNA in the serum (25, 26). In many patients, the rate of damage caused by the re-infection of HCV in the transplanted liver is faster compared to the HCV-infected non-transplanted livers, and the rapid formation of fibrosis and development of cirrhosis are common within the 9-12 years median after receiving the donor liver (4, 11, 24, 25). Also, about 75% - 80% on average suffer from relapsing chronic hepatitis (25). The actual determinants that are involved in the stimulation of HCV recurrence and progression are presently unclear (28).

The possibility of a relationship existing between the first detectable onset of HCVrec in the transplanted liver to the patient and graft survival rates have been investigated (27). It has been reported that the earlier the virus re-infects the liver allograft, the poorer the prognosis becomes for the patient (27). There have also been reports of HCV RNA tracing in the serum as soon as 48 hours post-liver transplantation (post-LT) and the severity of CHC becomes more aggressive in the re-infected livers (25, 29). The increase of aggression may be associated with improved immunosuppressive drugs that have been modified to become more effective in lowering the host's immune system to prevent rejection of the graft but giving the virus the upper hand to freely proliferate uncontrollably in the host (25). Moreover, a recent publication showed that the actual amount of immunosuppressant drugs, rather than the particular drug itself, affected HCVrec. Patients on double or triple immunosuppressant therapies commonly expressed

severe fibrosis or cirrhosis compared to the patients who were only on a single regimen (10). Overall, it is a difficult balance between preventing graft rejections while containing the viral infection so further studies are needed to explore other mechanisms. However, the role of immunosuppressant drugs and HCVrec severity post-LT is under discussion.

The initial cycle of re-infection has been thought to most likely occur during the reperfusion phase of the tissue and in majority of the time, these are same viral strain that infected the liver prior to transplantation (29-31). Therefore, some have begun to focus on pre-transplant antiviral treatment to possibly reduce the recurrence of HCV (10). Only a small percentage of HCV-related cirrhotic patients, however, actually responded to the treatment (demonstrating serologic HCV clearance before transplantation), and even a smaller subset within that group displayed no signs of HCVrec (10). Also, about 1/3 of the enrolled patients were not even able to complete the entire duration of the treatment and almost 2/3 of the patients needed a dose reduction due to the severe side effects and low tolerability (10).

The course of HCVrec is unpredictable, ranging from benign to severe among different cases, and many factors, such as the characteristics of the virus, donor, and recipient can all contribute to the progression of HCV (4, 25, 28). Research groups have even noted that certain cytokines, which mediate the immune response, differ in levels among patients with recurrent HCV compared to non-recurrent patients. In a recent study, the authors observed that IFN-γ expression levels were reduced to almost half in patients with recurrent HCV and those who had re-infection within 1 year after transplant had even lower levels of IFN-γ compared to those who developed the re-infection later (11). The

concept of low levels of IFN-γ triggering inadequately low amounts of T-helper 1 (Th1), which then leads to a decreased antiviral activity within the host, causing higher viral load and further progression of HCV to occur, could partially explain why HCVrec generates such rapid proliferation and aggressiveness in post-LT (11, 32). Studies have shown that within 5 years of liver post-LT, 30% of the re-infected patients will develop progressive fibrosis and graft cirrhosis and about 10% will need a second transplantation in order to survive (29, 30). Current antiviral therapies have shown limited out-come in treating HCVrec in post-LT and re-transplantation for HCV recurrence has demonstrated unfavorable results (10, 29). A study suggested that patients would have better results if re-transplantation was scheduled immediately after the first signs of HCV re-infection, instead of waiting until the their conditions worsen, to avoid further complications in surgery such as other organ failures (10). Although the logic may ideally be true, the reality is that there simply are not enough donor organs available for patients to undergo multiple organ transplants. Therefore, robust predictors of severe disease need to be identified in order to prevent or minimize impact on allograft function.

MicroRNAs

In 1993, Victor Ambros and his group came across an unusual gene, named the lin-4 gene, during their genetic analysis in Caenorhabditis elegans (C. elegans) (33-35). The lin-4 gene did not encode a protein but rather it produced a small non-coding RNA, (later given the name lin-4) that was roughly 22 nt in length. It was found to repress gene expression through imperfect binding of complementary sequences on target messenger

RNAs (mRNAs) which was necessary for normal development of the temporal region in C. elegans (33, 34). It was later realized that lin-4 carried out its regulation by binding to the 3'-UTR of the mRNA lin-14, which was one of the mRNA targets of lin-4 identified by Wightman (35). The interaction between lin-4 and lin-14 blocked translation, thereby regulating the transition of developmental timing of larval stage 1 (L1) to larval stage 2 (L2) (34).

About 6 years after the discovery of lin-4, a second small RNA named let-7 was noticed by the Ruvkun lab which was observed to target another developmental timing gene, lin-41 in C. elegans (33). Let-7 RNA was interestingly found to be evolutionary conserved across the animal phyla, from sea urchins to humans (33). This lead to the findings of lin-4 and let-7 being the first of many to follow be identified as the original microRNAs (miRNAs).

MiRNAs are small (roughly 18-25 nucleotides) noncoding RNAs that have been initially transcribed from miRNAs precursor genes by RNA polymerase II (pol II) as a ~200 nt long primary miRNA (pri-miRNA). It gets processed by the cellular RNase III enzyme Drosha inside the nucleus into the precursor miRNA (pre-miRNA) which is a ~70 nt loop structure with a 2-nt 3'-overhang that gets exported out of the nucleus by Exportin 5 (Exp5). The processed pre-miRNA then enters the cytoplasm where another cellular RNase III enzyme called Dicer further cleaves the miRNA into the mature 18-25 nucleotide miRNA:miRNA* duplex. Dicer also unwinds the two strands and the strand with the lower base pairing stability (called the guided strand) gets incorporated into a multi-protein complex called the RNA-induced silencing complex (RISC) where it can

now regulate gene expression through posttranscriptional modification of many target gene products The other strand (called the guide passenger strand) may have some functions but it is usually degraded (35-39).

MiRNAs can originate from either cellular or viral transcripts and bind in a sequence-specific manner to numerous target genes, causing either a cleavage of mRNA through perfect base pairing or a repression of mRNA translation through imperfect base pairing (2, 40). A single miRNA can target multiple mRNAs, and their targets are determined by the region known as the seed sequence, which involves perfect complimentary base-pairing of 5-8 nt between the target gene and the matching miRNA (35, 39, 42). Some miRNAs are found to be expressed randomly throughout the body whereas others are found to be expressed in a tissue-specific manner (2).

Since its discovery, the perception of gene regulation has evolved and the conservation of these miRNAs found within plants, various animals, and even in viruses, suggest ancient functions (34, 35). Currently, about 97% of the human genome is made up of non-coding deoxyribonucleic acid (DNA) in which 1% of the human genes have been estimated to encode miRNAs (35). These miRNAs can regulate up to 1/3 of the human genes that encode for proteins and their roles in controlling various processes are numerous, including cell proliferation, differentiation, apoptosis, and even interaction between virus and host cells (34, 35, 41). The miRNAs' diverse roles are continually expanding the knowledge in our understanding of the biochemical and bio-molecular aspects of the human body and these findings are opening up new approaches in medicine.

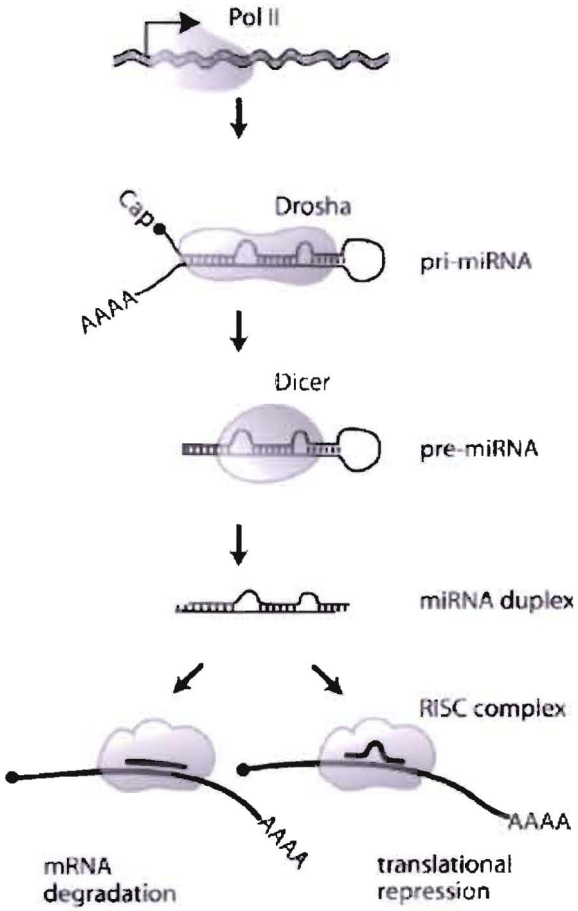


Figure-3: Illustration of the steps and proteins involved in the process of microRNA-dependent gene regulation. (T. Pietschmann, Journal of Hepatology 2009) (42).

MicroRNAs and HCV

Since HCV replication is predominantly located in the liver, the viral life cycle appears to be influenced by factors mainly found in the liver cells. There are expression patterns of mammalian miRNAs such as miR-122, miR-152, miR-194, miR-199, and miR-215 which have been discovered or predicted to be located specifically within the liver (36). Recent studies have suggested that miR-122 (which is endogenously produced in the

host cells) play a role in the stimulation of HCV RNA synthesis in liver cells and in the cultured human hepatoma Huh7 cells (1, 2, 18, 40, 43-45). MiR-122 is known to be abundantly made in the liver where it makes up about 70% of the total miRNA population and it is transcribed from the non-coding polyadenylated RNA transcript of the CCHCR1 gene (coiled-coil alpha-helical rod protein 1) which is thought to be a regulator in liver development (1, 2). Through bioinformatics, miR-122 is predicted to target hundreds of different mRNAs and its' regulatory roles are still being investigated (41).

In relations to miR-122 and HCV, prior experiment of mutagenesis has suggested that miR-122 binds to two sites on the 5'-UTR of HCV RNA, located upstream of the HCV IRES (2). These two sites lie next to each other with a short spacer in between them and the complementary matching of miR-122 to these binding sites, which is conserved among all six HCV genotypes, demonstrated a positive effect on the viral replication (2). High levels of miR-122 portrayed an accumulation of HCV translation while at low levels of miR-122, (which were achieved through antisense oligonucleotides) it resulted in an 80% decreased production of viral RNA genome in the liver cell lines Huh7 (2, 18). However, there was another study performed in which they gathered their data from liver biopsies of patients with CHC undergoing treatment and their findings suggested no positive association between HCV RNA levels and miR-122 (45). The role of miR-122 on the replication of HCV may have demonstrated more strongly in vitro compared to in vivo experiments but either way, conclusions drawn from in vitro studies should be carefully analyzed before assuming it as fact for in vivo applications (45).

There are other reports stating a group of cellular miRNAs, (miR-196, miR-296, miR-351, miR-431, and miR-448) when triggered by interferon, behaved as inhibitors of HCV replication. These miRNAs displayed almost a perfect match on the HCV RNA genome and actively lowered mRNA of HCV by 80% (44). Another recent study used a miRNA target search algorithm to identify miR-199a* having complementarity to the HCV genome in domain II of the IRES region in the 5'-UTR (46). They discovered that miR-199a* behaved like a RNA silencer and overexpression of this miR-199a* inhibited HCV replication, regardless of the HCV genotype. The suppression was irreversible by either mutation in the miR-199a* sequence or in its' target sequence in the HCV (46). These findings illustrate the versatile functions of miRNAs in the role of HCV. Some are known to enhance replication of HCV whereas others are known to inhibit the virus. The exact biological pathway in the involvement between these certain miRNAs and HCV however, still remains unknown.

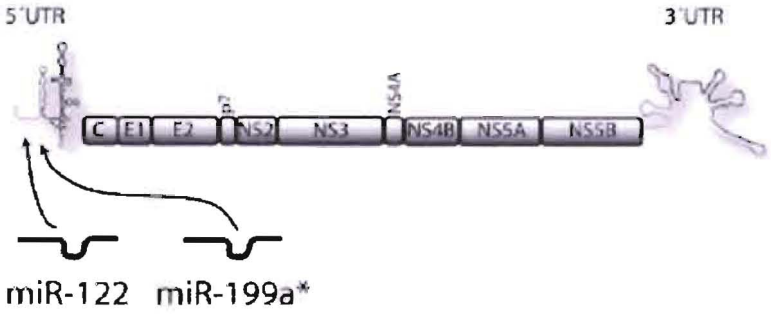


Figure-4: Schematic diagram of the HCV genome. The target sites for miRNAs known to regulate HCV RNA replication. (T. Pietschmann, Journal of Hepatology 2009) (42)

BCAP31 – A Predicted Target Gene of miR-122

BCAP31 (also known as BAP31) is an evolutionarily conserved, integral endoplasmic reticulum (ER) membrane protein, originally identified as B cell receptor-associated proteins (47-50). It is 28-kDa in length, with three transmembrane domains (TMDs), and functions as a cargo receptor to regulate intracellular trafficking and ER export of some transmembrane proteins (47, 48, 50). Some known roles of BAP31 are its ability to bind directly to cystic fibrosis transmembrane conductance regulator (CFTR) to control its surface expression in epithelial cells, its ability to bind to major histocompatibility complex class I (MHC-I) proteins to regulate its recruitment to transport vesicles, and its ability to regulate intracellular trafficking and cell surface expression of the leukocyte β_2 integrin CD11b/CD18 (47, 49, 50). It has been noted that in neutrophils, CD11b/CD18 are mainly located in secondary granules during inactivation and only during cellular activation, can it be translocated to the plasma membrane. The expression of these integrins on the plasma membrane of neutrophils allows them to make contact with ligands along the endothelial cells, so they can adhere and migrate to infected sites where it can carry on phagocytosis and pathogen invasion (47). BAP31 has been suggested to be involved in this up-regulation of the CD11b/CD18 on the surface of neutrophils which are the immune cells involved in the first line of defense against pathogens. BAP31 has also been shown to maintain the expression of CD9 and CD81 integrins on cell surfaces, playing an indirect role in cell adhesion and survival (50).

Several studies have also demonstrated that BAP31 is part of the ER quality control compartment and can cycle back and forth between the peripheral ER and the juxtanuclear

region (which are ER-associated compartments that differ from the ER-Golgi route). It interacts with the ER-associated degradation (ERAD) substrates to make sure that only properly folded proteins are delivered to their destinations while any inefficiently folded proteins gets retrotranslocated, ubiquitinated, and degraded by proteasomes located at the juxtanuclear region. Once the ERAD substrates has been assisted to its' designated location, BAP31 separates from the defect proteins and gets recycled back to the peripheral region to repeat the cycle (48).

In addition to these numerous roles of BAP31, another interesting role is its involvement with apoptotic proteins. In its original length, BAP31 is known to have anti-apoptotic activity but once the Fas death pathway is stimulated, caspase-8 becomes activated and cleaves the cytoplasmic end of BAP31 upstream of the mitochondrial death pathway. This blocks the BCL-2 anti-apoptotic pathway and the by-product of BAP31 cleavage, p20, stimulates rapid Ca2+ release from the ER, sensitizing the mitochondria to pro-apoptotic signals (50). p20 also remains in the ER where it interferes with uncleaved BAP31 and disturbs its ability to maintain cell surface expression of certain integrins (50).

Since the BCAP31 gene was listed as one of the predicted target sites of miR-122 and it has many important functions in maintaining vitality of the cells, it was chosen to be investigated to find any relations it may have with the expression levels of miR-122 and the severity in HCVrec. Due to the lack of research in the association of BAP31 and HCV, their relationship remains unknown. Maybe BAP31 modulates expression of certain integrins on the surface of host cells which in turn could contribute to the entry of HCV and result in cell to cell infectivity.

Moreover, in a recent study performed in our laboratory (51), using genome wide gene expression analysis in patients with HCV that underwent liver transplantation, BCAP31 was identified as significantly differentially expressed in patients that developed fibrosis at 3 years post-liver transplantation. BCAP31 was 3.3 fold changes over expressed in liver tissue samples at HCVrec time in HCV-transplant patients that progressed to severe fibrosis at 3 years post-LT when compared with HCV-liver transplant recipients that did not.

Involvement of CD4+ T cell response in relation to HCV infection

The CD4 gene encodes for the CD4 protein which are found on T lymphocytes. Major functions of CD4+ T-cells are stimulation of antiviral immune response through the release of specific cytokines such as Interleukin-2 (IL-2) and Interferon-γ (IFN-γ), assistance in the increasing production of B-cells antibodies, and maintaining memory CD8+ T-cells (52). In acute HCV infected individuals, studies have demonstrated that mounting an early, strong, multi-specific CD4+ T cell response to HCV non-structural proteins often lead to control of viral replication and spontaneous clearance of the virus (52-54). In contrast, patients with chronic hepatitis and persistent infection were frequently seen with inefficient amounts and short lived CD4+ T cells, correlating to the failure of viral clearance (52). In liver transplanted recipients, immunosuppressant drugs which interfere with the normal host immune response have shown an indirect influence over the severity of HCV recurrence (52).

The exact mechanism behind why some patients have deficient HCV-specific CD4+ T cell response compared to other patients are not clear. Several groups have suggested the idea that suppression of T cell response could be brought on by CD4+/CD25+ regulatory T-cells (Treg) (55, 56). Treg are another group within the T cells that function to regulate chronic immune activation, resulting to immunological tolerance (56). Normally, Treg are designed to prevent autoimmunity and excess host tissue damage from immune response towards pathogens. A healthy balance between a strong and efficient immune response to clear the virus while containing the activity of the immune system and inflammation is important. In chronic viral infections, the Treg initial goal of preventing further liver damage by reducing the immune response could possibly be helping the virus remain persist since HCV specific T-cell responses are being suppressed (56). The pathways behind the balance of Treg roles in HCV are currently unknown.
In our recent study (51), our findings support the hypothesis that HCV persistence and disease progression after OLT may be related in part to the lack of an appropriate helper T-cell response, whereas, in contrast, a vigorous T-cell response during the early stages of re-infection may be an important mechanism to limit liver allograft injury.

Study Rationale

Inhibiting the progression of severity in HCVrec is an important process to the outcome of the patient's survival but the exact biological changes that are responsible for the different transitional stages of liver fibrosis within individuals are presently unknown. Therefore, searching for molecular markers through the analysis of gene expression can contribute to the understanding of HCV infection and potentially be used to as an early predictor of severe recurrence. We hypothesize that the elucidation of associations between miRNA-122 and the histological severity in HCVrec post-LT might serve as a biomarker in predicting the outcome of HCV recurrence severity in patients and improve in the selection of liver transplant candidates that need antiviral treatment post-LT.

Research Aims

(1) To identify associations between the miR-122 patterns and the histological severity of HCVrec in liver transplant recipients.

(2) To identify associations between the BCAP31 patterns and the histological severity of HCVrec in liver transplant recipients.

(3) To identify associations between the CD4 patterns and the histological severity of HCVrec in liver transplant recipients.

CHAPTER 2 Materials and Methods

Patients. Our study involved 43 Caucasian patients who all underwent liver transplantation due to HCV cirrhosis. Each patient had liver biopsies taken at two time points: (1) at the time of HCVrec which is defined by detectable levels of HCV RNA in the serum and elevated (alanine aminotransferase) ALT within one year of the transplantation, and (2) at three years post-transplantation. Using the METAVIR histological scores (57), the patients were categorized into three groups: 1- Benign (36.4%), 2- Intermediate (31.8%), and 3- Severe (31.8%). According to the Metavir Score System, fibrosis is scored as F0 (absent), F1 (portal fibrosis), F2 (portal fibrosis with few septa), F3 (septal fibrosis) and F4 (cirrhosis). In addition, necroinflammation activity (A) is graded as A0 (absent), A1 (mild), A2 (moderate) or A3 (severe).

The study also included 10 normal liver biopsies which were taken from donor livers and served as the control group. Liver function and histopathology for these liver donors were shown to be normal. All 10 patients were seronegative for HCV antibody.

Six HCV infected, non-transplanted, cirrhotic liver samples were also included to observe for differential expression of the miR-122 and the genes between normal livers and the non-transplanted HCV infected cirrhotic livers to exclude immunosuppressant influences.

Liver specimens. Total RNA was isolated from formalin-fixed paraffin-embedded (FFPE) liver biopsies using RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion). Ambion® recommended using slices that are at least 10 μm thick in order to risk miRNA loss because anything thinner than 10 μm would cause cells to split open and if cytoplasm is revealed, miRNA can be washed away during the deparaffinization washing step. To determine how many tissue slices should be cut per sample, a test set of samples were sectioned into 2 slices and 4 slices. We wanted to observe which slices would be most efficient in extracting the most RNA out of the samples without clogging the filter from too much paraffin. After noticing that the 4 slices did not overwhelm the filters, we decided to increase it to 5 slices in order to maximize the collection of RNA for all the samples. The sectioning of the liver biopsies that were embedded in the paraffin blocks were performed by VCU Molecular Diagnostics (MDx).

RNA isolation. RNA isolation was performed using the Applied Biosystems (AB) RecoverAll™ Total Nucleic Acid Isolation Kit – Optimized for FFPE (paraformaldehyde-fixed, paraffin-embedded) samples/tissues. [Part Number AM1975] © *2008 Ambion, Inc.,* in accordance with the manufacturer's protocol. This kit was chosen for the study because it is specifically designed to extract total nucleic acids (RNA, miRNA, and DNA) from FFPE tissues and according to its' manufacturer, the recovered products are suitable for quantitative real-time RT-PCR.

The formalin-fixed and paraffin-embedded liver sections of 10 μm thickness were incubated at 50°C in xylene solution to initiate the de-paraffinization step. Once the paraffin melted and separated from the tissue samples, several washes with ethanol were carried out in order remove the excess xylene from the sample and accelerate drying of the tissue. Digestion buffer and protease was then added and incubated at 50°C/80°C to break and free the covalent bonds between the proteins and the nucleic acids. Then a series of rigorous washing steps was necessary to purify the nucleic acids as it gets captured on the glass-fiber filter. Additional steps of washing were performed to ensure full recovery of smaller RNA fragments (<200 nt) from the samples. The isolated RNA was stored immediately at -80°C freezer until needed for later analyzing.

Measurement of RNA quantity and quality

To determine the concentration and quality of RNA in each of the sample, spectrophotometer readings were analyzed. 1 μl of RNA isolation from each sample was diluted in 9 μl of Nuclease free water, and 5 μl of the diluted sample was aliquotted into TE (10 mM Tris-HCl) for preparation of spectrophotometer readings using the Spectronic GENESYS5. The absorbance reading at 260 nm was used to calculate the RNA concentration and the ratio of A_{260}/A_{280} absorbance readings were used to measure the level of RNA purity. Typically, the ratio of 1.8 – 2.1 would be the ideal values for these isolated samples. The ratio of A_{260}/A_{270} absorbance readings were used to observe for any protein contamination.

MicroRNA studies

We used the TaqMan® MicroRNA Assays and the manufacture's protocol on all the isolated RNA samples to detect and quantify mature miR-122. The reverse transcription (RT) involved the conversion of total RNA to cDNA using the reagents from TaqMan® MicroRNA Reverse Transcription Kit along with the specific looped RT primers from the TaqMan MicroRNA Assays (hsa-miR-122 and RNU 44). 20µl reaction mixtures were carried out in the thermal cycler (ABI 9700; Applied Biosystems, Foster City, CA, USA) at conditions set to 16°C for 30 minutes (incubation period for primer-RNA template anneal), 42°C for 30 minutes (reverse transcriptase activity period), 85°C for 5 minutes (reverse transcriptase inactivation period), and held at 4°C for infinity. Only 1µl of the isolated RNA samples was used to synthesize the RT products in order to conserve the RNA samples. RNU 44 was selected as the endogenous control for the study since it is known to be expressed universally across 38 tissues samples (including liver) at relatively stable and abundant expression levels (58). After a series of RT product dilution tests were evaluated, it was determined that the best approach would be to dilute the RT products that had no detectable levels of RNA from the spectrophotometer readings to 1:5µl and for the samples with detectable RNA levels to 1:15µl.

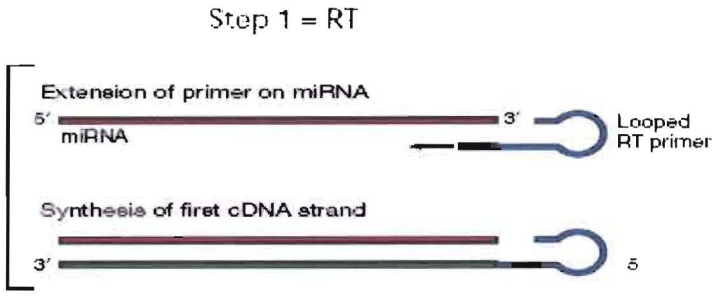


Figure-5: Reverse Transcription Step (TaqMan MicroRNA Assays Protocol 2006) (59)

TaqMan RT-PCR (Real Time - Polymerase Chain Reaction) was carried out for the amplification step of the PCR products from the cDNA, using the TaqMan MicroRNA Assay and the TaqMan® Universal Master Mix without AmpErase UNG (uracil-N-glycosylase). The purpose of UNG is to prevent PCR carryover products by hydrolyzing any uracil-glycosidic bonds to block DNA polymerase replication. However, due to our RNA samples being fragmented and degraded from the FFPE process and being aged, it was decided to exclude the AmpErase UNG from the Master Mix so we could maximize what little RNA that remained in our samples. 2.4µl of the diluted RT products was added to the 17.6µl of the reaction mixtures. The ABI Prism 7700 Sequence Detector was set at conditions of 95°C for 10 minutes (denaturation step), 92°C for 15 seconds (annealing step), and 60°C for 60 seconds (extension step) which was repeated for 40 cycles. The amplifications for miR-122 were performed in duplicates while the endogenous control RNU 44 was performed in single reaction.

What makes this assay robust from any other PCR is that TaqMan uses a sequence specific probe that anneals to the complementary sequence of the interested target site. The

probe contains a FAM™ reporter dye at the 5' end and a nonfluorescent quencher (NFQ) at the 3' end, followed by a minor groove binder (MGB) which prevents probe lengthening. When the probe binds to its' complementary sequence in between the forward and reverse primer sites during PCR, the distance between the reporter dye and the quencher dye prevents any florescence activity. However, once the DNA polymerase begins synthesizing the new strand and reaches the annealed probe, it will cleave the probe, free the reporter dye from the quencher dye, and the separation will cause the reporter dye to fluoresce. The ABI Prism 7700 Sequence Detector will capture the fluorescence signal, indicating that the target of interest has been detected and amplified.

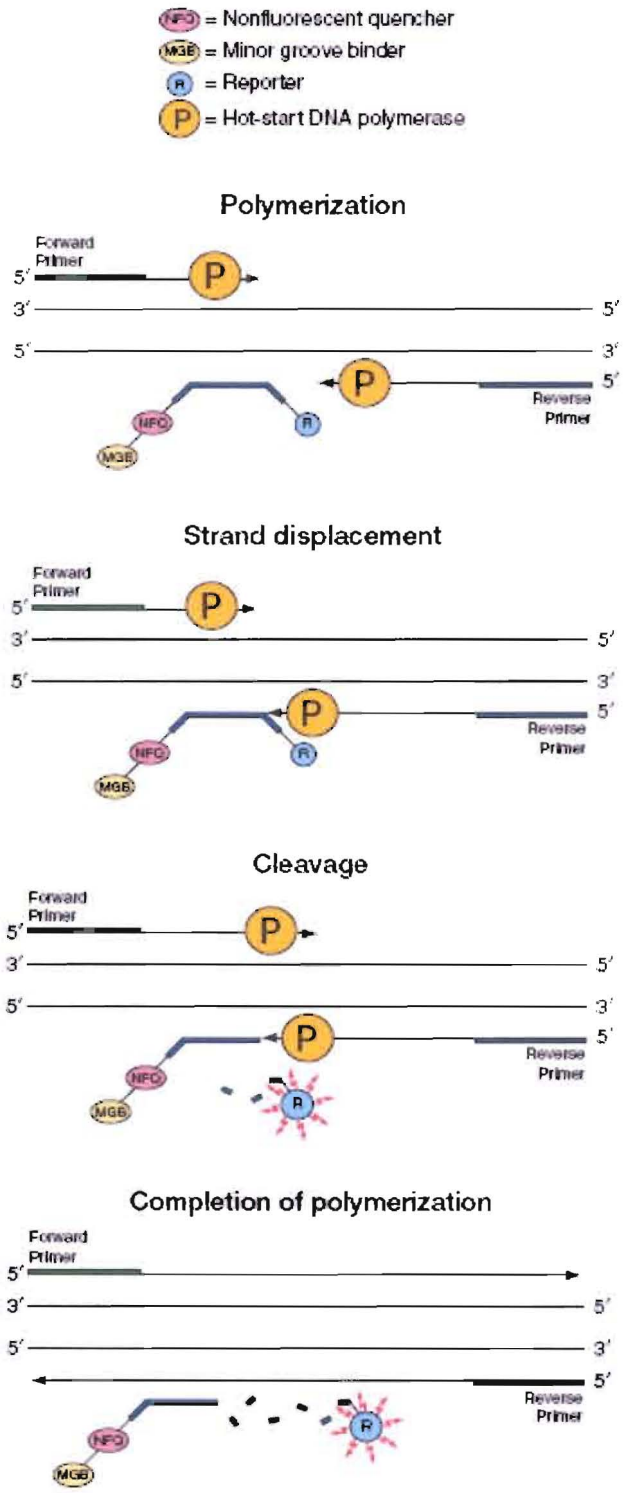


Figure-6: The PCR Step (TaqMan MicroRNA Assays Protocol 2006) (59)

The results portrayed amplification curves and the Critical Threshold (Ct) values for each reaction. The duplicate Ct values for each sample were averaged. No template controls (NTC) were also included in each run to ensure no false-positive had occurred. The Ct value of the miR-122 was subtracted from the Ct value of the RNU 44 to give the first ΔCt value. Delta-delta calculations were carried out to evaluate the fold change between the control group and the group of interest.

Gene expression studies

A list of 237 differentially expressed genes (DEG) (bead-types with a false discovery rate (FDR<10%) were considered significant genes) within selected RNA samples was obtained from a previously performed Illumina reaction when gene expression profiles were evaluated for the same patient group (51). The list of DEG was then compared to the lists of predicted gene targets of miR-122 that were gathered from three different database sources: MIRANDA, PicTar, and Target Scan. In accordance to the three databases, there were 40 genes in the DEG list that were found to be predicted targets of miR-122. The 40 DEG were further narrowed down to their functions, particularly focusing on those genes involving in fibrosis progression, matrix deposition and production, apoptosis and/or immune response. BCAP31 [B-cell receptor-associated protein 31] was selected to test out its expression in our samples. The gene CD4 was also added into the study because of its' important involvement in immune response against HCV. Studies have found that HCV recurrent infection may possibly behave in a similar

manner as acute HCV infection, suggesting the need for a strong and long-lasting effect of CD4+ T cell response for clearance and control of the virus (60). Defect in the cellular immune response has been associated with HCV persistence, leading to chronic infection (61).

TaqMan® Gene Expression Master Mix along with the TaqMan Gene Expression Assays was used following the manufacturer's protocol to detect and quantify BCAP31 and CD4 in the RNA samples. All the RNA samples were calculated and diluted to 8ng/µl and15µl of the normalized RNA was then aliquotted into 35µl of the reaction mixtures. Reverse transcription (RT) was carried out in the GeneAmp® PCR System 9700 at conditions set to 25°C for 10 minutes (incubation period for efficient primer-RNA template binding), 48°C for 30 minutes (reverse transcriptase activity), 95°C for 5 minutes (reverse transcriptase inactivation period), and held at 4°C for infinity using TaqMan® Reverse Transcription Reagents and random hexamers as the primers for conversion of total RNA into cDNA.

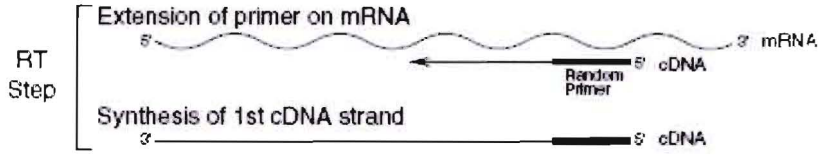


Figure-7: Reverse Transcription Step (TaqMan® Gene Expression Master Mix 2007) (62)

TaqMan RT-PCR (Real Time - Polymerase Chain Reaction) was performed next to amplify the PCR products from the cDNA by using the TaqMan® Universal Master Mix without AmpErase UNG along with the TaqMan® Pre-Developed Assay Reagents

Hs00271737-m1 BCAP31, CD4 and the housekeeping gene beta-2-microglobulin (B2M). The BCAP31 and CD4 assay contains a forward and a reverse unlabeled primers along with a FAM™ dye-labeled TaqMan® MGB probe while the housekeeping gene B2M also contains two unlabeled primers but differs in that its' probe is labeled with a VIC™ reporter dye.

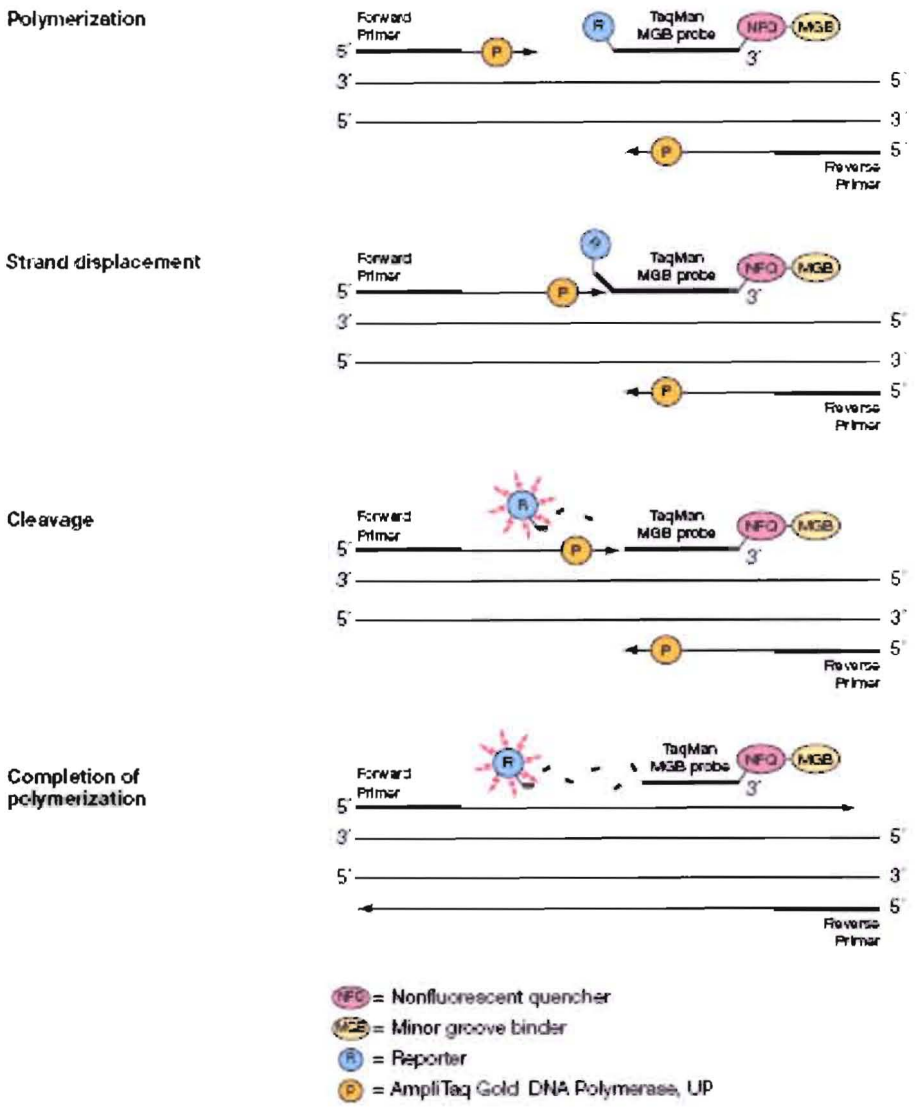


Figure-8: Chemistry Overview of 5' Nuclease Assay (TaqMan® Gene Expression Master Mix 2007) (62)

Beta-2-microglobulin (B2M) was selected as the housekeeping gene for the study because a test set demonstrated favorable Ct value in the FFPE samples compared to glucuronidase-beta (GUSB) which is another housekeeping gene that was recommended as a suitable reference gene for analyzing liver tissue infected with chronic hepatitis (63). GUSB and B2M were tested on four RNA isolations, two from FFPE samples and two from frozen liver tissues. The Ct values suggested GUSB would work well as the housekeeping gene for analysis of RNA from frozen tissues however, for the FFPE samples, B2M was the better choice because of the lower Ct value, implying better amplification (Table 1).

Table 1. Ct Values for the Two Housekeeping Genes:

	Sample #	GUSB	B2M
Frozen Liver Samples:	D-587	25.14	22.13
	D-597	25.35	23.26
FFPE Liver Samples:	5512	33.73	29.10
	15818	34.88	30.18

PCR was carried out in 20µl reaction mixtures in the ABI Prism 7700 Sequence Detector at conditions set to 95°C for 10 minutes (denaturation step), 92°C for 15 seconds (annealing step), and 60°C for 60 seconds (extension step) which was repeated for 40 cycles. All amplifications for the genes of interest were carried out in duplicate reactions, along with a single reaction of the endogenous control B2M beside each run. The levels of B2M mRNA were used as the endogenous reference for normalization to quantify BCAP31 and CD4. No template control (NTC) was run with every reaction to ensure proper assay function.

The results portrayed amplification curves and the Critical Threshold (Ct) values for each reaction. The duplicate Ct values for each sample were averaged. The Ct value of the genes of interest was subtracted from the Ct value of the housekeeping gene B2M to give the first ΔCt value. Delta-delta calculations were carried out to evaluate the fold change between the control group and the group of interest.

Amplification efficiency test

To validate the $2^{-\Delta\Delta Ct}$ calculation, amplification efficiencies of the target (miR-122 and BCAP31) and reference (RNU 44 and B2M) must be equal to each other. Therefore, the efficiencies were evaluated on 1:5 serial dilutions for the target gene and reference gene. The slopes of $\log_{10}$ (1/dilution factor) versus Ct were calculated for each reaction.

Statistical analysis

Student t-tests were used to compare the expression of miR-122 and the genes between Group 1 (Benign) to Group 3 (Severe) using their ΔCt values ($\Delta Ct = Ct_{(target\ gene)} - Ct_{(endogenouse\ control)}$). ANOVA tests were used to compare the expression levels of the genes and miR-122 between all three groups. Pearson's Correlation Analysis was used to observe for correlations between (1) miR-122 expression and viral load and (2) miR-122 expression and BCAP31 expression. Pearson's Correlation Coefficient of ±1 indicates strong correlation. For all three of the tests, $P<0.05$ were considered significant.

CHAPTER 3 Results

Patients

The study included 43 adults who underwent OLT for HCV-cirrhosis at Favaloro Foundation in Buenos Aires, Argentina, 8 of which (17%) had hepatocellular carcinoma. Mean's recipient age was 53.5±8.0 years and 58% (25 out of 43) were males. Nine patients (21%) were grafted with live donors. Donor age was 37.5±15.1 years for deceased donors and 33.7±10.2 years for live donors. The majority of patients (36 out of 43, 83.7%) were infected with HCV genotype 1. After OLT, recipients were immunosuppressed with calcineurin inhibitors (29 (67.4%) cyclosporine A, 14 (32.6%) tacrolimus, and corticosteroids which were withdrawn during the first year in 39 (91%)). Seven patients received induction therapy (4 OKT3, 1 ATG, 2 basiliximab) and 33 (77%) a third immunosuppressive agent at some time during follow-up (28 mycophenolate, 5 azathioprine). The prevalence of acute cellular rejection and treated acute rejection was 51% and 44% respectively. Patients with acute rejection received one IV bolus of 1 gram of methylprednisolone followed by an oral recycle with a starting dose of 200 mg/day of prednisone.

Mean follow-up post-OLT was 8.3±2.6 years. All patients underwent liver biopsies at HCVrec time, as defined by detectable HCV RNA in serum and elevated ALT, at 1 year

of OLT, annually thereafter and when clinically indicated. The median number of biopsies per patient was 6 (range 3-14). Based on histological analysis by METAVIR score (34) at 36 months post-OLT patients were allocated to 3 groups according to the severity of allograft fibrosis: 1) Group 1: mild fibrosis (F0-F1, n=16), 2) Group 2: moderate fibrosis (F2, n=14) and 3) Group 3: severe fibrosis (F3-F4, n= 13). No patient received antiviral therapy for HCV during the complete duration of the study. Time-to-clinical HCVrec was 5.0±3.8 months after post-OLT. Liver biopsies were obtained at clinical HCVrec time in 41 patients and showed acute hepatitis in 8, chronic hepatitis in 18 and minimal inflammatory changes in 15. In two patients with persistently normal ALT after OLT the first biopsy was performed by protocol at 1 year and in both cases it showed chronic hepatitis. Genome wide gene expression was initially analyzed in 12 patients (24 samples) and subsequently validated in the remaining 31 patients (58 samples) [Table 2].

Table-2. Characteristics of Liver Transplant Recipients

N	43	Group 1	Group 2	Group 3
Age (yo)	53 ± 8	51 ± 8	53 ± 9	56 ± 7
Gender (% males)	58	69	43	62
Virus genotype (% Genotype 1)	84	94	57	100
HCC (% present)	17	13	29	15
Donor Type (% Deceased Donors)	79	81	87	69
Immunosuppressant drugs (% CsA)	67	56	64	85
Treated acute rejection (%)	44	31	57	46
Follow-up period post-transplantation (years)	8.3 ± 2.6	9.6 ± 2.5	7.6 ± 2.5	7.5 ± 2.4
Mean time of HCVrec (months)	5.0 ± 3.8	5.6 ± 4.8	5.7 ± 3.7	3.7 ± 1.9

MiR-122 Expression

The differential expression of miR-122 between normal livers from normal liver donors *versus* cirrhotic livers from patients with CHC infection was first evaluated. Two sample t-tests were performed to evaluate the expression of miR-122 within normal livers compared to non-transplanted (non-TX), HCV infected cirrhotic livers [Figure 9]. Each sample contained a miR-122 Ct value and an endogenous control RNU 44 Ct value. The ΔCt values, which is equivalent to the difference in threshold cycles for target and reference ($Ct,x - Ct,R$) for normalizing the expression miR-122 were analyzed. The t-tests value of $P= 0.0006$ showed significant differential expression between the two groups. $2^{-\Delta\Delta Ct}$ calculations were carried out to demonstrate relative fold changes in miR-122 expression (which were initially normalized to the endogenous reference gene RNU 44)

between the control group (normal livers) and HCV infected livers (non-TX). There was a pattern of down-regulation in miR-122 expression in HCV infected livers compared to normal livers, fold changes < 0.2. Amplification efficiencies for miR-122 and RNU 44 using the 1:5 serial dilutions had slopes of -3.59 and -3.58 respectively [Figure 10], indicating similar amplification and validating the use of the delta-delta method.

Next, we aimed to evaluate the expression of miR-122 in HCVrec post-LT to observe for differential expressions between the groups of histological severity (classified using the METAVIR score in the biopsy at 3 years post-LT). T-tests were performed on the mean ΔCt values between Group 1 (Benign) and Group 3 (Severe) and we found no significance in the level of miR-122 expression at each time point, (P= 0.95 at time of recurrence, P= 0.29 at 3 years post-transplantation (post-TX), respectively) [Figure 11]. Even though the difference between the two groups are not considered to be statistically significant, we do notice a pattern of miR-122 expression increasing at 3 years post-TX compared to at time of HCV recurrence.

An Analysis of Variance (ANOVA) was further applied to the data set, and the results were consistent with the previous t-tests, confirming no significance in expression of miR-122 between the two groups. The ΔCt values of miR-122 were used as the outcome variable and the severity groups were used as the dependent variable [Table 3].

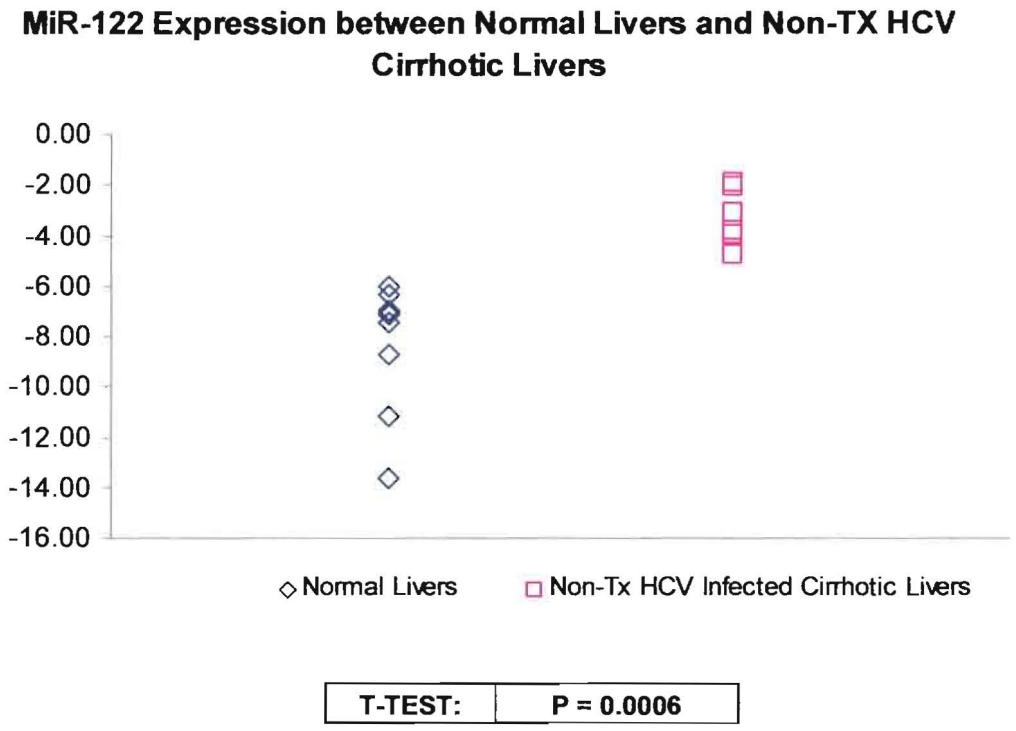
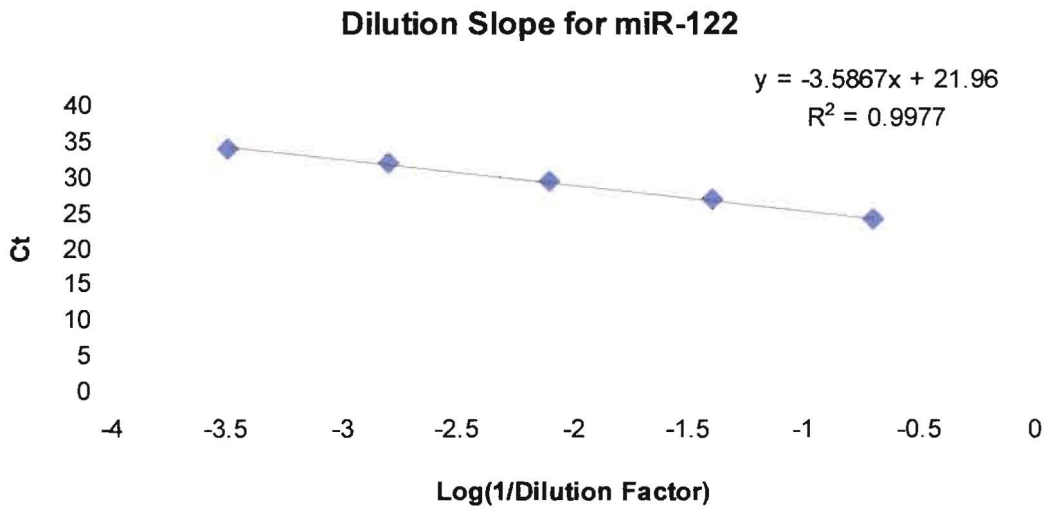


Figure - 9. Comparing the miR-122 ΔCt values between normal liver samples and in HCV infected liver samples that were not transplanted.



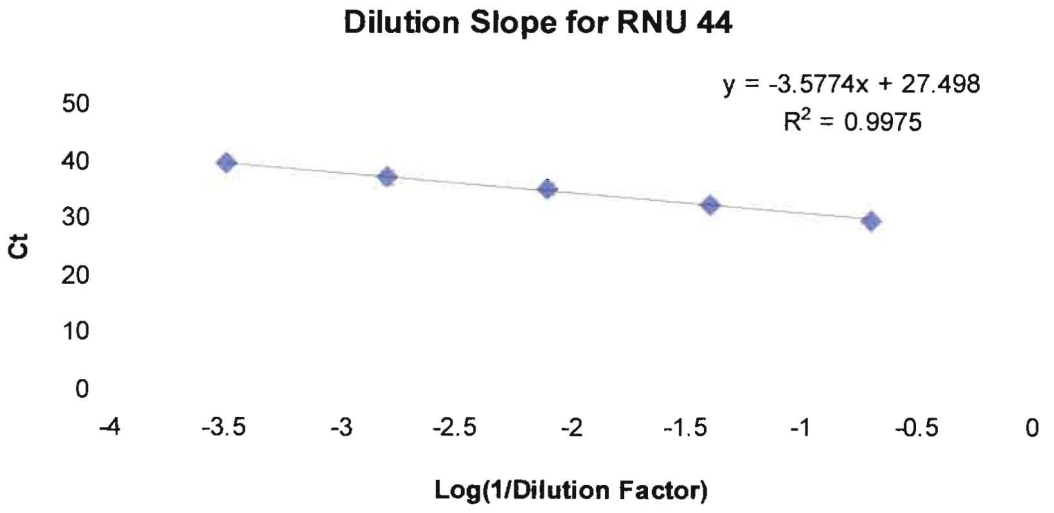
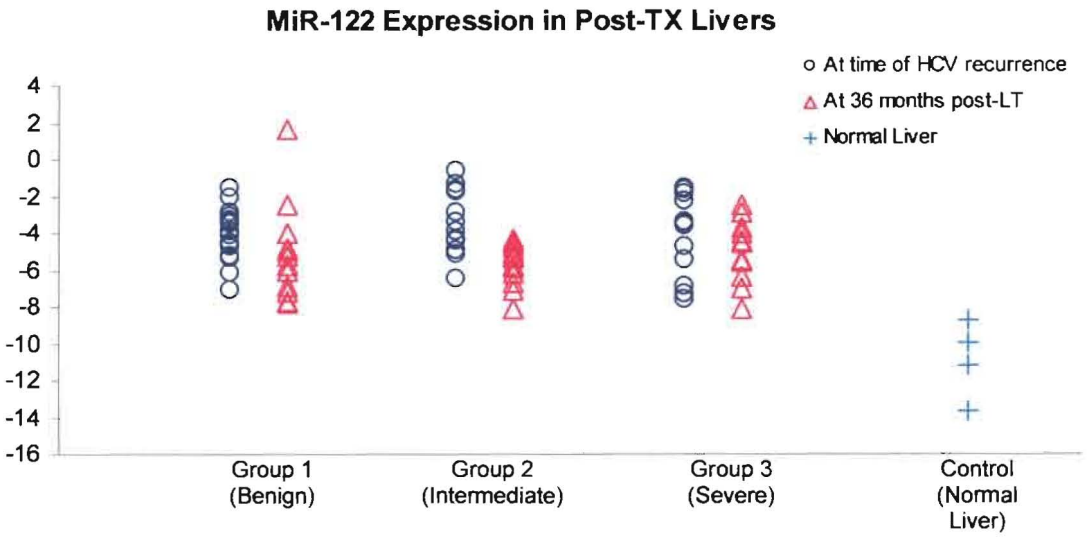


Figure-10. Dilution Slopes for miR-122 and RNU 44 using the 1:5 serial dilutions.



At time of HCV recurrence:	**GROUP 1 vs 3 T-Test**	**P = 0.95**
At 3 years Post-Tx:	**GROUP 1 vs 3 T-Test**	**P = 0.29**

Figure - 11. Delta Ct values of miR-122 in HCV recurrence groups.

ANOVA Results

A

Comparisons significant at the 0.05 level are indicated by ***.			
Recurrence Comparison	**Difference Between Means**	**Simultaneous 95% Confidence Limits**	
Group 3 (Severe) - Group 1 (Benign)	7.324	-6.416	21.064
Group 3 (Severe) - Group 2 (Intermediate)	9.726	-4.447	23.899
Group 1 (Benign) – Group 2 (Intermediate)	2.402	-11.065	15.869

B

Comparisons significant at the 0.05 level are indicated by ***.			
Recurrence Comparison	**Difference Between Means**	**Simultaneous 95% Confidence Limits**	
Group 3 (Severe) - Group 1 (Benign)	-2.493	-12.400	7.414
Group 3 (Severe) - Group 2 (Intermediate)	-3.838	-14.062	6.386
Group 1 (Benign) – Group 2 (Intermediate)	-1.344	-10.801	8.113

Table 3. Comparison of the outcome variable Δct miR-122 within groups: (A) at time of HCV recurrence and (B) at 3 years post-TX.

MiR-122 and Viral load

To observe for any correlation between the level of miR-122 expression and the amount of virus in the bloodstream (viremia), a Pearson's Correlation Analysis was applied to the data. The results in [Table 4] suggested a positive correlation in Group 3 (Severe) at time of HCVrec.

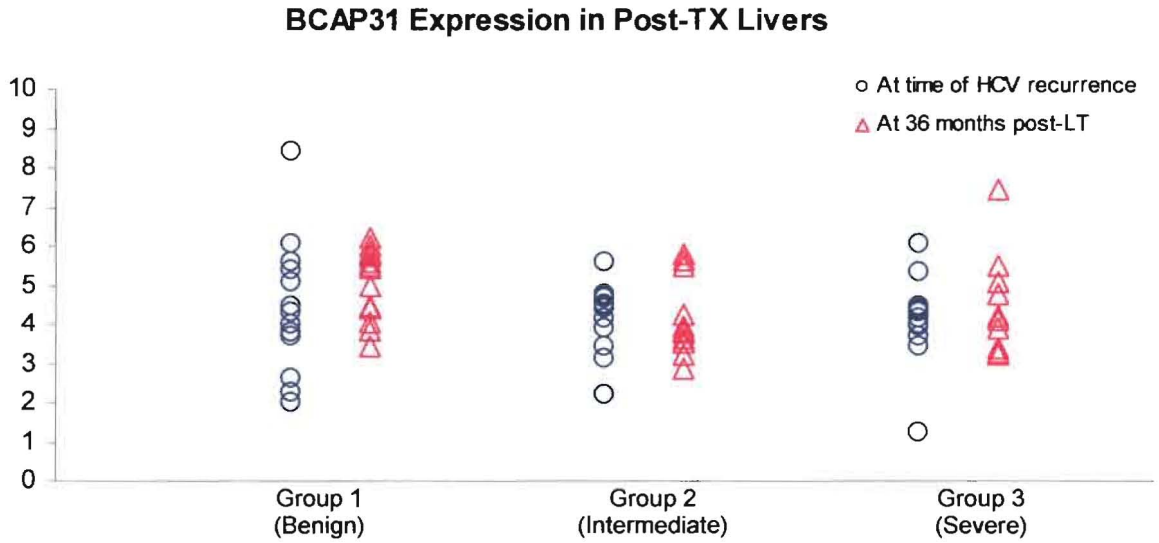
TABLE 4. Pearson's Correlation Coefficient Values for miR-122 and Viremia

Recurrence group levels	Pearson Correlation Coefficients(* denotes a significant correlation)	
	Time of HCV recurrence	Three years post treatment
Group 1 (Benign)	0.03	0.08
Group 2 (Intermediate)	-0.35	0.41
Group 3 (Severe)	0.58*	-0.37

BCAP31 Expression

To investigate the expression patterns of BCAP31 among the severity groups, the student t-tests on the mean ΔCt values between Group 1(Benign) and Group 3 (Severe) at each time point were carried out. There were no significance found in the level of BCAP31 expression among the two groups (P= 0.49 at time of recurrence, P= 0.09 at 3 years post-TX) [Figure 12]. Delta-delta calculations were also performed to evaluate the relative fold changes in the expression of BCAP31 (which have been normalized to the endogenous housekeeping gene B2M) between the control group (normal livers) and Group 1 (Benign) and 3 (Severe) independently. There were no significant patterns of BCAP31 expression

among the groups. Amplification efficiencies for BCAP31 and B2M using the 1:5 serial dilutions had slopes of -3.80 and -3.47 respectively [Figure 13].



At time of HCV recurrence:	**GROUP 1 vs. 3 T-Test**	**P = 0.49**
At 3 years Post-Tx:	**GROUP 1 vs. 3 T-Test**	**P = 0.09**

Figure - 12. Delta Ct values of BCAP31 in HCV recurrence groups.

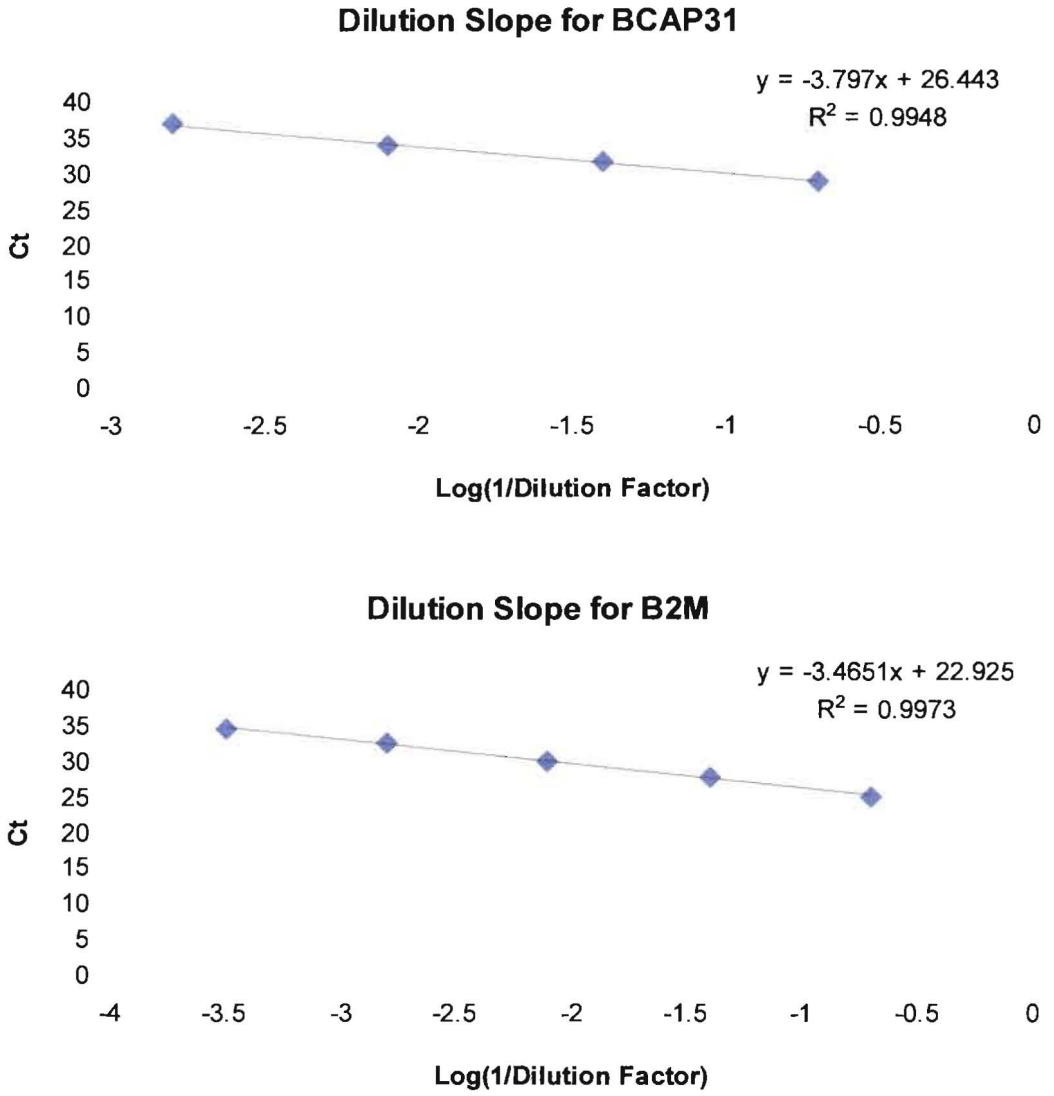


Figure-13. Dilution Slopes for BCAP31 and B2M using the 1:5 serial dilutions.

BCAP31 and miR-122 Expression

Since BCAP31 was an in silico predicted target of miR-122, we wanted to observe for any correlation between the expression of BCAP31 and the expression of miR-122 within each group at each time point. We used Pearson's correlation coefficient and found

that there is one significant correlation between BCAP31 and miR-122 only at the time of HCVrec in Group 3 (Severe). It appears to have a negative relationship. The rest of the groups had no significant correlations at either time points.

TABLE 5. Pearson's Correlation Coefficient Values for miR-122 and BCAP31

Recurrence group levels	Pearson Correlation Coefficients(* denotes a significant correlation)	
	Time of HCV recurrence	Three years post treatment
Group 1 (Benign)	0.32	-0.20
Group 2 (Intermediate)	-0.14	0.07
Group 3 (Severe)	-0.56*	0.22

CD4 Expression

To evaluate the expression of CD4 in regards to cirrhotic fibrosis, t-tests were done on the mean value of ΔCt between the normal liver samples (6.69) and the non-TX HCV infected cirrhotic liver samples (6.95). The student t-tests value of $P= 0.669$ suggested no significance [Figure 14].

To observe the role of host cellular specific immune response in the progression of HCVrec, we evaluated the CD4 expression at time of HCVrec. The student t-tests revealed $P= 0.053$, suggesting differential expression of the mean ΔCt values between Group 1 (Benign) and Group 3 (Severe) at time of HCVrec being statistically significant [Figure 15].

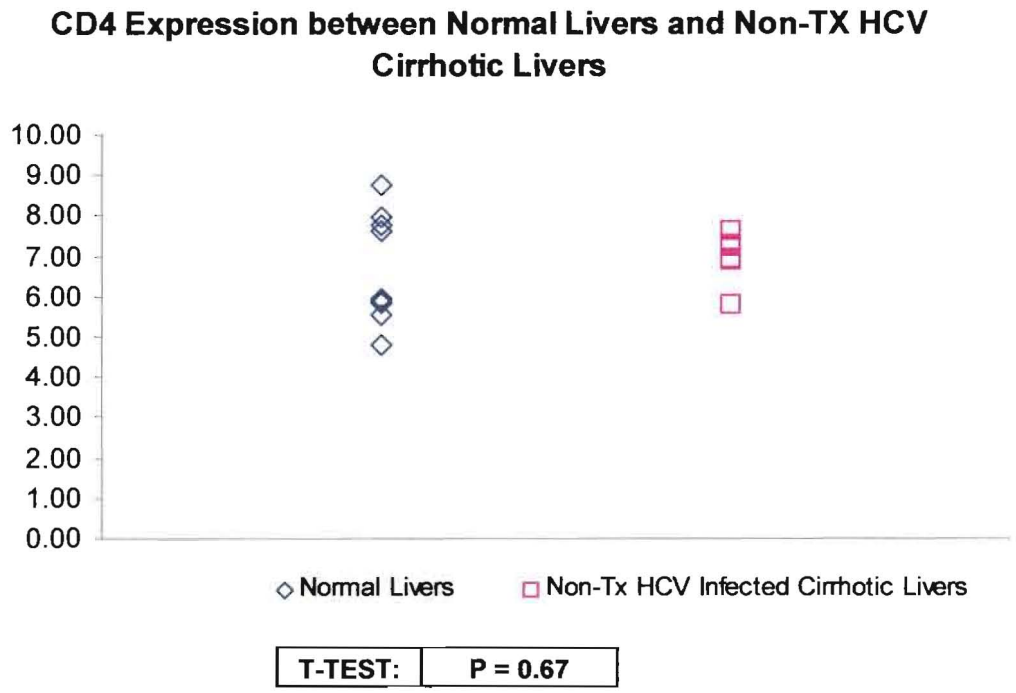


Figure - 14. Comparing the CD4 ΔCt values between normal liver samples and in liver cirrhotic samples from patients with chronic HCV

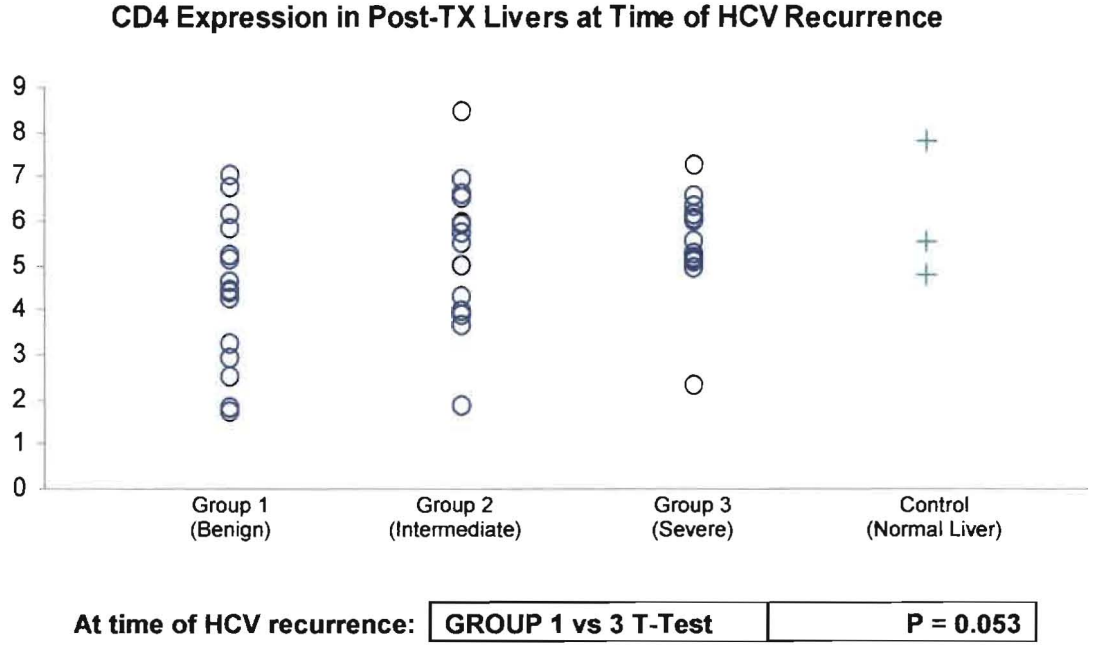


Figure - 15. Delta Ct values of CD4 in HCV recurrence groups.

CHAPTER 4 Discussion

Hepatitis C Virus infection is one of the major leading cause of liver diseases, infecting approximately 3% of the world population in which 80% of that population fail to eradicate the virus, resulting in the life-long battle of chronic infection (64). Liver transplantation is the current treatment to the end-stage liver disease and re-infection is universal. The progression of the disease is unpredictable and each case results in different diagnosis, ranging from benign to severe in the development of fibrosis. Due to the complexity of the host-virus interaction, the causes behind this wide range of differences in the outcome of the patients are still being investigated. In our study of patients with HCV recurrence, 36.4% maintained a mild form of the infection whereas 31.8% progressed on to histological fibrosis within 36 months after transplantation. Little progress has been made in the management of HCVrec in order to decrease the risk of graft loss and need for re-transplantation. Strategies to improve management of HCV recurrence include not only the availability of more effective antiviral agents but also the identification of robust predictors of severe disease in order to prevent or minimize the impact on allograft function.

There have been several studies that have reported miR-122 playing a positive role on the replication of HCV in cell culture, particularly in Huh 7 cells (40, 43, 68). MiR-122 is known to be abundantly produced specifically in liver cells (~66,000 copies per cell) and

Jopling et al. have demonstrated that sequestering miR-122 in Huh-7 cells by antisense oligonucleotides lead to a decrease of HCV RNA up to 80% (43). In addition to these findings, there have been other studies that were able to demonstrate miR-122 involvement in the stimulation of HCV translation by enhanced interaction between the small ribosomal subunit with HCV RNA (18, 68).

In contrast to other indications for OLT, the recurrence of HCV infection (as defined by detectable HCV RNA) is an almost universal phenomenon. Typically, serum levels of HCV RNA increase rapidly from week 2 post OLT, peaking at 1-3 months, achieving 1-year post-OLT levels that are 10-20-fold greater than the mean pre-LT levels. Viremia at 1-year post transplantation usually plateaus at 1-2 logs higher than pre-transplantation levels (29). Although significant improvements have been made in outcomes after OLT during the past 15 years, little progress has been made in the management of post transplant hepatitis C. The majority of patients with virological recurrence will develop histological graft injury, usually chronic hepatitis.

In the present study, we aimed to evaluate associations between miRNA-122 expression in liver allograft biopsies and histological severity of HCV recurrence post-LT. From the analysis of our data, we observed differential expression of miR-122 between normal livers and HCV infected cirrhotic livers. As it was previously mentioned, miR-122 is known to be abundantly produced specifically in liver cells. This finding might indicate involvement of miR-122 in HCV infected livers and was the initial justification for the study.

When we further evaluated differences in expression levels of miR-122 among transplant HCV patients with different degree of histological fibrosis severity in the allograft at 3 years post-LT, our data did not show significant expression between the three groups. However, we did find positive correlations between the expression of miR-122 and viral load which is in agreement with Jopling et al. results. Viral load is considered a contributing factor to the severity of the disease because the rapid increase of viral loads may possibly lead to the exhaustion of proper priming and maturation of cell-mediated immune response (25, 60). In majority of patients suffering from chronic HCV infection, HCV persistency is often seen with inefficient immune response whereas the absence of detectable HCV RNA in serum in patients who have achieve sustained viral response through the use of antiviral therapy resulted in significant improvements in the histological severity, consequently inhibiting the progression of the disease (60, 68, 69). Higher HCV RNA levels (serum and liver) at the time of LT have been associated with enlarged risk of progression to cirrhosis, graft loss, and death. In our patient group, viral loads were high for all the patients at pre-LT time. Higher viral load levels in the early post-LT period have also been associated with more rapid progression (70, 71). These results have encouraged studies of early pre-emptive antiviral therapy in an attempt to delay HCV recurrence in the allograft. However, the benefit of use of anti-viral treatment after LT is unclear. In our study group, all the patients maintained an important viral load post-LT. The patients included in this study did not receive anti-viral treatment post-LT. and as consequence, we evaluated normal course of disease progression. Even when all the patients presented continue high viral load (pre-LT and post-LT) there was high variability in the course of

HCV recurrence progression, indicating the complexity and multi-factorial nature of the disease.

Since microRNAs are involved in the regulation of numerous gene expression in the host by repression of mRNA translation, we have selected the gene BCAP31 (which was listed as a predicted target of miR-122) to investigate the expression pattern in the HCV recurrence groups. BCAP31 is known to regulate intracellular trafficking and ER export of some transmembrane proteins (including the expression of MHC-I), contributing to the stability of host cells. An in vitro study demonstrated a reduction of MHC-I expression on the surface of HCV infected cells, possibly being one of the viral strategy of avoiding detection from the host immune surveillance by interfering with the process of antigen presentation (61). BCAP31 could possibly be involved indirectly in the process of reduced MHC-I expression on infected cells.

A previous study done by our lab using Whole Genome Gene Expresion (WGE) DASL (Illumina), reported a 3.3 fold change over expression in patients who developed fibrosis at 36 months after transplantation (51). In our study, we did not find BCAP31 expression being statistically significant between the three groups. However, a negative statistical significantly correlation was observed between the expression of miR-122 and BCAP31 for the patient group that progressed to severe fibrosis at 3 years post-LT. This correlation supports the computationally predicted target pair relationships between the miRNA and mRNA. Since miRNAs are known to regulate gene expression through the binding of their target genes in a perfect or imperfect complementary sequence, resulting to

a degradation or repression of mRNA, our findings of an inverse relationship between miR-122 and BCAP31 support our assumptions of their interaction (42).

There have been numerous studies investigating the role of immune response in hepatitis C viral infection and many of them suggest that a strong and multi-specific CD4+ T cell response against the virus coincides with controlling and clearing the infection (69). Therefore, it has been implied that a lack of CD4+ T cell results in deficiency in CD8+ T cell activity and impairment of antibody production, which may contribute to chronic infection (61). Although CD4 is not a gene targeted by miR-122, we were interested to observe the expression levels among our HCV recurrent groups. Our data portrayed statistically significant differential allograft expression of CD4+ between Group 1 (mild fibrosis) and Group 3 (severe fibrosis) at the HCV recurrence time. This finding supports the assumption that the lack of efficient CD4+ T cells may be contributing to the viral persistency and progression of the disease in post-transplantation. There have also been other studies that have demonstrated similar findings in which a poor and low CD4+ T cell response was correlated to the severity of recurrence in post-LT (65). Since CD4+ T helper (T_H) cells are known to be activated by the interaction of viral peptides presented on antigen presenting cells, leading to a cascade of cytokine release for recruitment of other immune cells to elicit antiviral activities for eradication of the virus, low expression levels of CD4 could possibly be a contributing factor to the progression of the disease (72).

Conclusion

Being able to identify advanced fibrosis at an early stage through the use of biomarkers would be beneficial for prevention of bad prognosis. In our study, we demonstrated that miR-122 expression had no significance in the severity of HCV recurrence but it did correlate with the amount of HCV viral load. Niepmann et. al demonstrated in living non-liver HeLa cells that miR-122 were involved in the acceleration and enhancement of the small ribosomal subunit interaction with the HCV RNA (68). Henke et. al demonstrated that the stimulation of HCV RNA translation were independent from viral RNA synthesis through the use of replication-defective NS5B polymerase mutant genome and were able to show the direct interaction of miR-122 at the 5'-UTR of HCV genome being responsible for the stimulation of HCV translation in liver cell lines and HeLa cells (18). Both these studies suggest miR-122 being involved in the initiation of HCV translation and our finding of a positive correlation between miR-122 and viral load supports these results.

Due to the miRNA characteristic of regulating the expression of numerous protein-encoding genes, our findings of an inverse relationship in the expression of BCAP31 and miR-122 is in agreement to our current understanding of the miRNA:mRNA interaction. The exact mechanisms in the binding interaction between miR-122 and BCAP31 are currently unclear.

An increase of miR-122 expression resulting in a decrease of BCAP31 expression might have an indirect role in the persistency of the virus because modulating the expression of BCAP31 can interfere with the vitality of host cell but the pathways are

presently unknown. Our research is the first step in the development of a non-invasive assay for prediction of severity of HCV recurrence. To fully understand the biology of miRNAs with specific diseases, miRNA:mRNA target pairs must be identified and confirmed. While our data are supportive of computationally predicted target pairs, they will need to be empirically verified through target specific expression, detection, and quiescence.

Reduction in levels of CD4 expression appears to be correlated with poor prognosis of the disease while an increase of CD4 in the early stages of recurrence is associated with positive outcome. Studies done in humans and chimpanzees illustrated a strong and multi-specific T cell response being seen with clearance of acute HCV infection while a lack of CD4+ T cell help was commonly observed in chronic infection (69). Exhaustion or anergy could possibly be involved in the reduction of CD4+ T cells response which is suggested to be associated with the persistency of the virus. Therefore, the role of host immune response to virus might play a critical role in HCV recurrence post-LT and deserves further evaluation.

Deciphering the molecular pathways involved in the initial events conducting to the development of fibrosis in HCV recurrence may in fact be impossible to interpret, when a graft with established injury is sampled. Thus, early genomic sampling of the graft post-OLT may be the approach most likely to result in the greatest yield for mechanisms or predictive factors. A set of biomarkers investigated early after LT, when both HCV re-infection and host response are ongoing in the allograft, may be used to predict whether a

patient will experience aggressive recurrence with accelerated fibrosis. Identification of such biomarkers may lead to a therapeutic approach that specifically target patients at risk.

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